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Isolation, characterization and mode of neutralization of a potent antihemorrhagic factor from the serum of the snake *Bothrops asper*

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Abstract

A potent antihemorrhagic factor (BaSAH[1]) was isolated from the serum of the snake *Bothrops asper* by ammonium sulfate precipitation at 40–60%, Sephacryl S-200 and Sephadex G-50 gel filtration, DEAE-Sepharose, and hydrophobic Phenyl-Sepharose chromatography. The purified protein showed one band with an isoelectric point of 5.2 and a molecular weight of 66 kDa. 4 μg of the purified factor BaSAH were needed to neutralize the hemorrhagic dose of *B. asper* whole venom compared to 60 μg of the clinically used horse polyvalent immunoglobulins. Moreover, 0.35 μg of BaSAH were sufficient to achieve complete neutralization of the main hemorrhagic toxin (BaH1), with a molar ratio of 2:1. The antihemorrhagic activity was stable between pH 1.5–9 and up to 60°C but lost activity completely after 30 min of heating at 70°C. BaSAH did not digest the hemorrhagic toxin BaH1 or formed a precipitin line with it, nor with the whole venom. Both ELISA experiments and chromatography of BaSAH after incubation with the ^{125}I-labeled hemorrhagic toxin BaH1 demonstrated that the mechanism of the neutralization involves a formation of an inactive soluble complex between the natural antihemorrhagin and the main hemorrhagin of *B. asper* venom.

Keywords: Antihemorrhagins; Venom; Serum; Neutralization

1. Introduction

The majority of snake bites in Central America are caused by pit vipers, especially by *Bothrops asper*. Hemorrhagic activity is considered one of the most relevant pathophysiological effects induced by this venom, as it develops quickly after venom injection causing major microvasculature damage and blood loss which leads to muscle and other tissue degeneration [1,2]. Similar striking consequences of envenomation, due to the occurrence of local and systemic hemorrhage, are caused by most crotalid and viperid snake venoms [3,4].

Recently, we isolated and characterized three hemorrhagic toxins from the venom of *B. asper*, BaH1 being the main one [5] and investigated the ultrastructural alterations induced by the whole venom [2] and by BaH1[6] in mouse blood capillaries.

The polyvalent antivenom produced from horses at the Instituto Clodomiro Picado in Costa Rica is currently being used as the basic therapeutic device for the treatment of snakebite envenomation in Central America. However, both clinical and experimental data indicate that the local hemorrhagic activity is only partially neutralized, even when the antivenom is administered rapidly after envenomation [7]. Moreover, there have been reports of severe allergy to horse serum contradicting the use of horse antivenom in some patients [8]. It is therefore important to search for other compounds which can effectively neutralize the hemorrhagic and other harmful activities of *B. asper* whole venom.

It is well established that certain snakes and mammals are resistant to envenomation by viperid or crotalid snakes [9], including some nonvenomous snakes [10,11]. Two different strategies of resistance to snake venoms have been found so far. The mongoose and several snakes resist Viperotoxin and alpha-bungarotoxin because their recep-

Abbreviations: BaSAH, *Bothrops asper* antihemorrhagic factor; SAS, saturated ammonium sulfate; BaH1, *Bothrops asper* main hemorrhagic toxin; MHD, minimal hemorrhagic dose; AMHD, minimal antihemorrhagic dose; PBS, phosphate buffered saline; PAGE, polyacrylamide gel electrophoresis; ELISA, enzyme linked immunoabsorbent assay

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tors do not bind the toxins [11–16]. Other animals develop natural antidotes in their plasma that neutralize various viperid and crotalid toxins [9,17,18]. The neutralization capacity of these natural factors is very high compared to specific immunoglobulins: up to 20 times higher in *Vipera palestinae* serum [12,17,19] and up to six times in the serum of *Crotalus atrox* as compared to the commercial polyvalent antivenom produced for clinical use in North America [20].

Among several snake and mammalian sera screened in this laboratory for their antihemorrhagic activity against the whole venom of *B. asper*, the homologous serum of *B. asper* itself was found to possess the highest neutralizing activity [21]. The antihemorrhagic activity was found in 40–60% saturated ammonium sulfate (SAS)[2] serum fraction. Moreover, this fraction neutralized very efficiently the hemolytic, lethal, proteolytic and cytostatic effect of the venom [22].

This work deals with the isolation, characterization and the mode of neutralization of a potent antihemorrhagin from the serum of *B. asper* (BaSAH), which may have a clinical application.

2. Materials and methods

2.1. Materials

Sephacryl S-200, Sephadex G-50 and G-25, DEAE-Sepharose, and Phenyl Sepharose CL-4B were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden; Acrylamide and N,N'-methylenebisacrylamide were obtained from BDH, Poole, U.K. ELISA plates were purchased from Nunc, Roskilde, Denmark. All other chemicals were analytically pure and were purchased from Merck, Darmstadt, F.R.G. or from Sigma Chemical Co., St. Louis, Missouri, U.S.A.

2.2. B. asper serum, venom and the main hemorrhagic toxin (BaH1)

The snakes were kept at Instituto Clodomiro Picado, University of Costa Rica. Blood was collected from several snakes and pooled; the serum was separated by conventional methods, lyophilized and stored at −20°C until used. Venom was 'milked' from several snakes, pooled, lyophilized and stored at −20°C until used. BaH1[3]—the main hemorrhagic toxin in the venom of *B. asper*—was purified as previously described by Borkow et al. [5].

2.3. Antisera

Antiserum against *B. asper* hemorrhagin 1 (BaH1) was prepared by injecting 1 ml of BaH1 (60 μg) mixed with 1 ml of Freund's complete adjuvant subcutaneously into rabbits. Three boosters were injected at one month intervals. The rabbits were then bled one week after the last booster and the serum was separated by conventional methods.

2.4. Determination of antihemorrhagic activity

The hemorrhagic activity of the whole venom and BaH1 was determined by the skin injection method of Kondo et al. [23] with some modifications of Ovadia [24]. One tenth of a milliliter of saline containing 0.36–20 μg of the examined material was injected into the skin of the back of white mice. Two hours later a red hemorrhagic spot was observed on the inner surface of the removed skin. The amount of venom or BaH1 that produced a hemorrhagic spot of about 1 cm in diameter was defined as one minimal hemorrhagic dose (MHD[4]). The MHD of the whole venom is 10 μg, and of BaH1 is 0.18 μg.

The antihemorrhagic activity was examined by incubating the serum or its fractions with 80 μg of *B. asper* whole venom or with 1.44 μg of BaH1 in 0.4 ml PBS for 1 h at 37°C. After incubation 0.1 ml of each mixture was injected intracutaneously into the skin of three white ICR mice anaesthetized with ether. Control included samples of the serum fraction or corresponding amounts of the whole venom or of BaH1 which were injected separately. The mice were sacrificed 2 h later and the inner surface of the removed skin was examined. The minimal antihemorrhagic dose (AMHD[5]) was defined as the minimal amount of protein required for complete neutralization of one MHD.

2.5. Thermostability and pH stability of the antihemorrhagin

The thermostability of the antihemorrhagic activity found in the whole serum or in the purified antihemorrhagin was tested at different temperatures as follows: 0.4 ml aliquots containing 12 AMHD of the purified BaSAH or the whole serum in PBS[6] were heated at various temperatures between 37°C and 100°C for 30 min and cooled before 1.8 μg (10 MHD) of *B. asper* hemorrhagin 1 (BaH1) were added to each aliquot. The mixtures were then incubated for 1 h at 37°C and the hemorrhagic activity was examined as above.

The stability of the antihemorrhagic activity at various pH values was tested by incubating aliquots containing 10 AMHD of the BaSAH for 1 h at various buffer solutions, followed by overnight dialysis against PBS, pH 7. Then the antihemorrhagic activity of each aliquot was examined as described above.

2.6. Digestion by trypsin

Samples containing 15 mg of *B. asper* serum in 1 ml PBS were incubated with 0.2% trypsin for 1 h at 37°C and soybean trypsin inhibitor was added (2% final concentration) in order to inactivate the trypsin. As control antihem-

orrhagin was incubated with PBS only or with PBS containing 2% soybean trypsin inhibitor. Each of these mixtures was then tested for antihemorrhagic activity as described above.

2.7. Polyacrylamide gel electrophoresis (PAGE[7])

Polyacrylamide gel electrophoresis (PAGE) was carried out according to the method of Davis [25]. The purified factor and the whole venom were applied on 10% polyacrylamide slab gels. The running buffer was 0.05 M Tris-glycine, pH 8.3 and a current of 20 mA was applied. The gels were stained with 0.025% Coomassie Brilliant Blue in 10% acetic acid containing 25% isopropanol. Destaining was carried out at room temperature by several changes of 10% acetic acid containing 10% isopropanol.

2.8. Determination of the molecular weight

Molecular weight was determined in a non-denaturating system as outlined by the Sigma Technical Bulletin No. MKR-137. Markers with known molecular weight and the examined proteins were electrophoresed on a set of polyacrylamide gels with various concentrations and the gels were stained with 0.025% Coomassie Brilliant Blue. Relative electrophoretic mobility [R_f] of each protein in each gel was determined according to the tracking dye and the 100[log($R_f \times 100$)] was plotted against the percent gel concentration for each protein. The logarithm of the negative slope was plotted against the logarithm of the molecular weight of each protein. The molecular weight of the antihemorrhagin was determined from the linear plot obtained.

2.9. Isoelectric focusing

Markers of known pIs (β-Lactoglobulin A from bovine milk, pH 5.1; Carbonic anhydrase II from bovine erythrocytes, pH 5.4; Carbonic anhydrase II from bovine erythrocytes, pH 5.9 and Carbonic anhydrase I from human erythrocytes, pH 6.6) and the antihemorrhagic fraction were run separately in polyacrylamide gels prepared as follows: the markers or the antihemorrhagic fraction were mixed with 2 ml of a 10% polyacrylamide solution containing 1% carrier ampholine with a pH range of 3–10. Each mixture was then poured into a tube and left under fluorescent light for 2 h to polymerize. 200 V were applied for 16 h and then 300 V for 2 h. The anode solution was 0.125% phosphoric acid and the cathode solution 0.25% NaOH. The gels were fixed with 10% TCA before staining by 0.025% Coomassie Brilliant Blue.

2.10. Ammonium sulfate precipitation

Cold saturated solution of ammonium sulfate was added to the serum in a cold room under continuous stirring until 40% saturation was achieved. After 30 min, the suspension was centrifuged for 20 min at 10,000 × g and the precipitate was redissolved in 0.01 M phosphate buffer, pH 7. Additional saturated ammonium sulfate was added to the supernatant to attain 60% saturation and 30 min later the suspension was treated as above followed by a dialysis against the same phosphate buffer. The antihemorrhagic activity of the fractions (0–40%, 40–60%, > 60%) was examined as described above.

2.11. Immunodiffusion

Immunodiffusion was performed in 1% agar gels according to the procedure of Ouchterlony [26]. The antihemorrhagic fraction was placed in the central wells and the whole venom or the purified hemorrhagic fraction was poured into the peripheral wells at a distance of 0.9 cm. Antibodies against *B. asper* venom were used as control. After overnight incubation at room temperature (20–25°C) the gels were washed with saline for 3 consecutive days, dried and stained with 0.5% Ponceau S dissolved in 5% trichloroacetic acid.

2.12. Radiolabeling of BaH1

Iodination of BaH1 was performed according to the method of Salacinski et al. [27] with some modifications of Borkow et al. [28]. Briefly, samples of 20 μg Iodogen dissolved in 50 μl of chloroform were dispersed at the bottom of each glass tube and evaporated to dryness at room temperature under nitrogen and kept in the refrigerator until used. 100 μg of BaH1 were mixed with 0.5 mCi of $Na^{125}I$ and incubated in the Iodogen tubes for 20 min at room temperature. The free iodine was separated from the mixture by gel filtration through a 10 ml column of Sephadex G-25 equilibrated with 0.01 M sodium phosphate buffer, pH 7.2.

2.13. Colorimetric determination of albumin

Determination of albumin content was done by using Sigma Diagnostics Albumin Reagent (Catalog No. 631-2) which contains bromocresol green. The calibration was done by using Protein Standard Solution, Catalog No. 540-10.

2.14. Complex formation between BaSAH and the hemorrhagic toxin BaH1

ELISA[8] plates were used in the following experiments with which 100 μl aliquots were incubated in each step for 30 min at 37°C; after each step the ELISA wells were washed five times with tap water and dried. The first step included incubation of 6 μg of the purified antihemorrhagic factor in 0.01 M sodium phosphate buffer, pH 7.2 with each well in the plate. After washing the wells, 2%

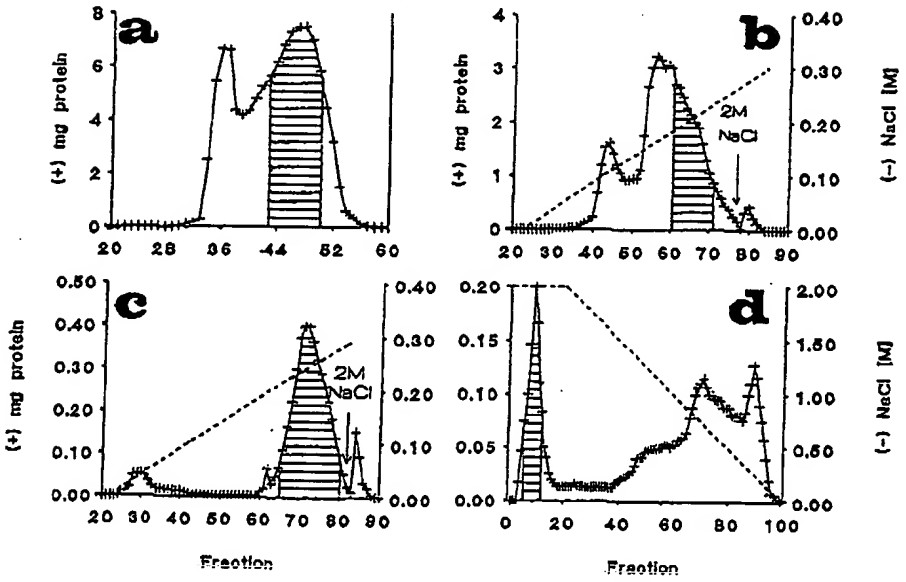

Fig. 1. Purification scheme: 500 mg whole serum were dissolved in PBS and the 40–60% saturated ammonium sulfate (SAS) serum fraction was collected. The antihemorrhagic activity was found in the shaded fractions of each purification step, which were pooled. a. Gel Filtration on Sephacryl S-200: The 40%–60% SAS fraction was applied to a column (2.3 × 90 cm) and eluted with 0.01 M sodium phosphate buffer, pH 7.2, at a flow rate of 2 ml/cm^2 per h and fractions of 4 ml were collected. b. DEAE-Sepharose chromatography, pH 7.2: The active fractions were applied to a 1 × 15 cm column equilibrated with 0.01 M sodium phosphate buffer, pH 7.2. The column was washed with the same buffer and developed with a linear salt gradient, followed by 2 M NaCl; the flow rate was 6 ml/h and 1 ml fractions were collected. c. DEAE-Sepharose chromatography, pH 8.3: Pool B was dialysed against 0.01 M Tris-HCl, pH 8.3, before it was applied to a 1 × 15 cm column equilibrated with the same buffer. After washing the column with the same buffer, a salt gradient was applied at a flow rate of 6 ml/h followed by 2 M NaCl; effluent was collected in 1 ml fractions. d. Hydrophobic chromatography: Pool C was mixed with NaCl to a final concentration of 2 M NaCl before it was applied to a 1 × 15 ml column of Phenyl Sepharose CL-4B equilibrated with 2 M NaCl. The antihemorrhagic fraction was eluted with 2 M NaCl and then a linear gradient between 2 M NaCl and water was applied.

bovine serum albumin (BSA) was incubated as a blocker. In the third step, ^{125}I-labeled BaH1 in 100 μl phosphate buffer was added to each well. Finally, after the washing, 200 μl of 1 N NaOH were added to each well in order to dissolve the proteins in the wells. Ten min later the radioactivity of the NaOH solution of each well was measured. As control, in the first step, only 0.01 M sodium phosphate buffer, pH 7.2 were used, without the antihemorrhagic factor.

^{125}I-labeled toxin (BaH1), the antihemorrhagin (BaSAH) and a mixture of BaSAH with the radiolabeled hemorrhagin BaH1 incubated at 37°C for 1 h were chromatographed separately on a 2.3 × 90 cm column of Sephacryl S-200. The column was eluted with 0.01 M sodium phosphate

Table 1
Isolation of BaSAH from the serum of *B. asper*

Purification step mg	AMHD (μg)	Total	AMHD	Purification factor
Whole serum	500	[a] 30	16667	1
		[b] 6	83333	1
40–60% SAS	178	[a] 13	13692	2
		[b] 2.8	63570	2
Sephacryl-200 [S1]	60	[a] 5	12000	6
		[b] 1.1	54545	5.45
DEAE-Sepharose pH 7 [D1]	6.6	[a] 3.8	1737	7.9
		[b] 0.8	8250	7.5
DEAE-Sepharose pH 8.3 [A1]	6	[a] 3.7	1620	8.1
		[b] 0.7	8570	8.6
Phenyl-Sepharose [BaSAH]	0.6	[a] 4	150	7.5
		[b] 0.35	1715	17.1

AMHD, antihemorrhagin neutralizing minimal hemorrhagic dose.
[a] The amount (μg) needed to neutralize one MHD of the whole venom of *B. asper*.
[b] The amount (μg) needed to neutralize one MHD of the purified hemorrhagic toxin BaH1.

buffer, pH 7.2. the flow rate was 15 ml/h and the effluent was collected in 4 ml fractions. The radioactivity and the protein content were measured in each fraction of the three chromatographs.

2.15. Proteolytic activity

General proteolytic activity was tested on azocoll according to the method of Moore [29] with a modification of Ovadia [30]. Briefly, aliquots containing 0.1 mg of the antihemorrhagic fraction were incubated separately with 10 mg of azocoll (insoluble dye -protein complex) suspended in 2 ml of 0.1 M sodium phosphate buffer, pH 7.2 in a shaking bath (120 strokes per min) for 16 h at 37°C. The reaction was stopped by centrifugation and the proteolytic activity was estimated by measuring the absorbance of the clear supernatant at 520 nm. PBS served as control.

Proteolytic activity on gelatin was examined according to Ovadia [24]. A drop containing 0.1 mg of the purified factor was applied to an undeveloped black and white film and incubated for 3 h at 37°C in a moist chamber. The film was then washed thoroughly with tap water. Gelatin digestion is indicated by a transparent spot on an opaque backround. Trypsin served as a positive control.

3. Results

3.1. Purification and properties of BaSAH

The purification procedure of BaSAH, the antihemorrhagin from the serum of *B. asper*, involved five steps which are detailed in Fig. 1 and Table 1.

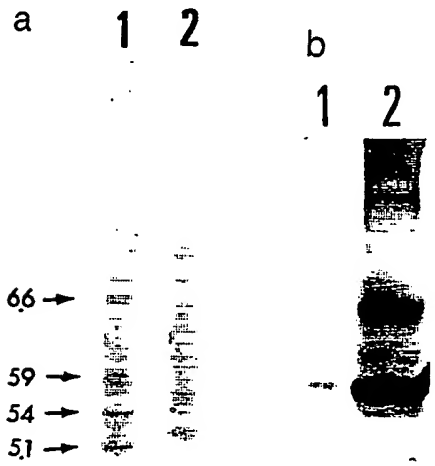

Fig. 2. a. Isoelectrofocusing: A mixture of four markers and BaSAH were run separately overnight at 200 V and then at 300 V for two hours in 2 ml of 10% polyacrylamide gels containing 1% carrier ampholines with a pH range of 3–10. Lane 1: four markers of known pIs; Lane 2: BaSAH. b. PAGE: 2 μg of the purified factor and 30 μg of the whole serum were run separately on 10% polyacrylamide slab gel. Lane 1: BaSAH; Lane 2: whole serum.

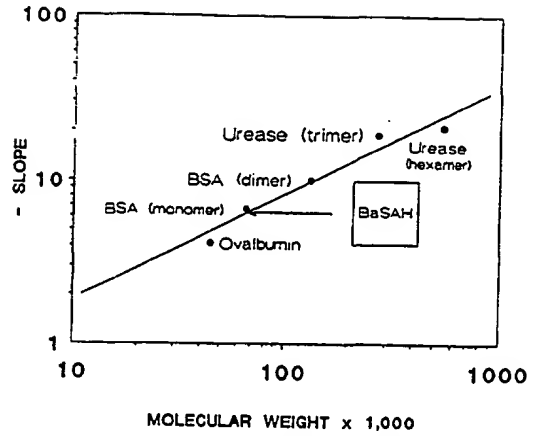

Fig. 3. Molecular weight determination: A. BaSAH as well as four molecular weight markers were electrophoreted on a set of gels of various polyacrylamide concentrations. From the relative mobility of each protein in each gel relative to the tracking dye the molecular weight of the antihemorrhagin was determined. (For details see 'Methods'). BaSAH showed one band with a mol wt. of 66 kDa.

BaSAH showed one band both in PAGE and in isoelectrofocusing with a pI of 5.2 (Fig. 2) and with a molecular weight of 66 kDa (Fig. 3).

Inactivation of the antihemorrhagic activity after incubation of BaSAH with trypsin indicated that the antihemorrhagic is a protein. BaSAH did not exhibit any proteolytic activity when tested on azocoll and gelatin (for details see methods section) and was stained by bromocresol green. The antihemorrhagic activity of the whole serum and purified BaSAH was stable between pH 1.5–9 for 1 h and up to 60°C for 30 min but lost activity completely after 30 min of heating at 70°C and

3.2. The mechanism of neutralization

SDS-electrophoresis of BaH1 after 1 h of incubation with the neutralizing factor BaSAH at 37°C did not reveal bands of low molecular weight, which indicates that the mechanism of neutralization does not involve an enzymatic cleavage of the hemorrhagic toxin BaH1 by the neutralizing factor.

Immunodifusion tests showed that neither the purified hemorrhagic toxin nor the whole venom of *B. asper* formed any precipitin line with BaSAH, in contrast to their precipitation with the polyvalent antivenom prepared in horses against the whole venom (not shown).

Separate chromatography of the labeled ^{125}I-BaH1 toxin and the neutralizing protein BaSAH on Sephacryl S-200 column showed elution of the toxin in the region of smaller molecular weight than that of the neutralizing factor (fractions 150 and 125 respectively, Fig. 4a.b). However, when the labeled toxin was incubated with the neutralizing protein before the chromatography on Sephacryl S-200, there was a clear shift in the elution volume of the radiolabeled toxin toward the region of

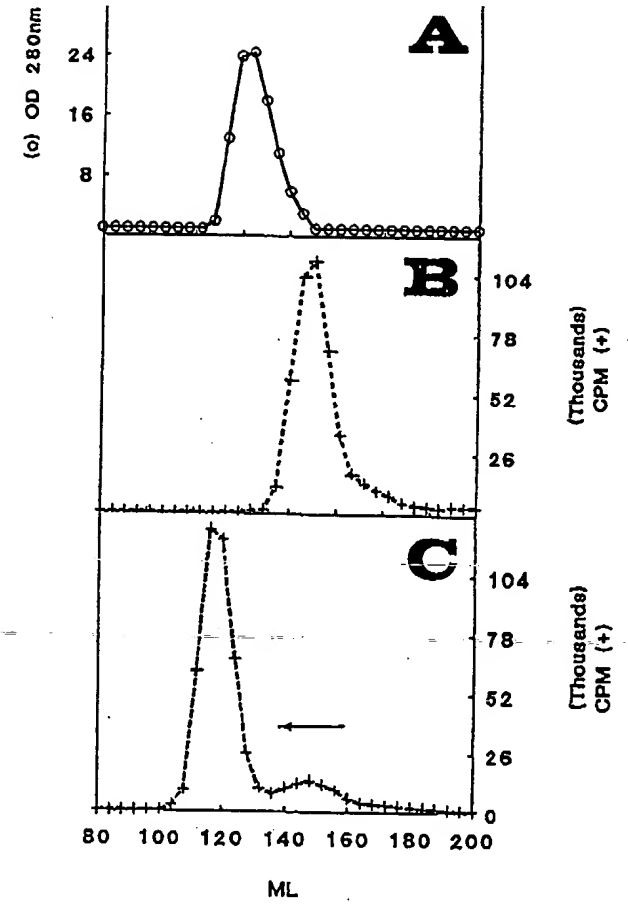

Fig. 4. Complex formation between BaH1 and BaSAH: Labeled hemorrhagic toxin [B] and the antihemorrhagin [A] were chromatographed separately on a 2.3×90 cm column of Sephacryl S-200. The column was developed with 0.01 M sodium phosphate buffer, pH 7.2, at 15 ml/h and the effluent was collected in 4 ml fractions. The radioactivity and the protein content were measured in each fraction. [C] -80 μg of BaSAH were incubated with the radiolabled hemorrhagin at 37°C for 1 h before the chromatography on the same column under identical conditions as above. Most of the labeled hemorrhagin was shifted to the region of the neutralizing protein, indicating the formation of a complex between the toxin and the natural antidote.

higher molecular weight, from fraction 150 to fraction 118 (Fig. 4c), thus suggesting that the neutralizing protein BaSAH forms a larger inactive but soluble complex molecule with the toxin BaH1.

Furthermore, experiments with ELISA plates demonstrated that BaH1 formed a complex with the antihemorrhagic factor BaSAH. The radioactivity of the control group (n = 8) was 52.5 ± 6.6, whereas those of the experiment (n = 5) were 70 ± 10; the p value is 0.0032 (two-tailed) as calculated by Student's t-test.

0.35 μg of the antihemorrhagin BaSAH with a molecular weight of 66 kDa neutralizes 0.18 μg of the hemorrhagic toxin BaH1, that has a molecular weight of 64 kDa. It may be concluded, therefore, that given the large effect of the 2:1 complex, there must be a very tight binding of at least one antihemorrhagin with the toxin resulting in a negative test.

4. Discussion

As *B. asper* is the most abundant and dangerous snake in Central America and South Mexico, we have screened in a recent work [21] various antisera and various inhibitors (natural and synthetic) that can effectively neutralize the hemorrhagic activity of *B. asper* venom. The homologous serum of *B. asper* possessed the highest neutralizing capacity, which was even higher than that of the isolated immunoglobulin fraction of the horse polyvalent antivenom currently used as the main therapeutic material. This work has therefore focused on the isolation and characterization of the main antihemorrhagic factor found in the serum of *B. asper*, here named *B. asper* Serum AntiHemorrhagin (BaSAH). Four μg of the purified antihemorrhagin BaSAH were sufficient to neutralize one hemorrhagic dose (MHD) of the whole *B. asper* venom in comparison to 60 μg of the horse polyvalent immunoglobulin fraction. Moreover, the equivalent fraction of the Bothrops serum (40–60% saturated ammonium sulfate) neutralized the major activities of the venom (hemorrhagic, lethal, hemolytic and cytostatic) more efficiently than many other examined compounds [22].

The molecular weight of the antihemorrhagic factor is 66 kDa, it is stained by Bromocresol green and it does not form precipitin lines with the hemorrhagic toxin BaH1 or the whole *B. asper* venom, which indicates that BaSAH is an albumin-like protein. This is in accord with previous findings showing that other antihemorrhagic proteins isolated from venomous and nonvenomous snakes [9,12,17,20,31,32] as well as from mammalian sera [33–38] were also non-immunoglobulins but rather albumin-like factors [9,13,17,39,40].

Although the mechanism involved in the process of neutralization of the snake toxins by the natural antidotes attracted the attention of the investigators, it was studied for a few antidotes only. So far, this laboratory has examined the neutralization mechanism of the lethal Viperotoxin (from *Vipera palaestinae*) and shown that the neutralizing protein formed an inactive complex with one of the synergistic components of the toxin [19]. A mechanism of complex formation was also reported for additional natural antidotes [32,39,41,42].

BaSAH did not exhibit any proteolytic activity and did not digest the toxin. A complex formation between the toxin BaH1 and the natural antidote was demonstrated by ELISA experiments and by the shift of the radiolabeled toxin when it was preincubated with BaSAH before it was applied onto a molecular sieve chromatography (Fig. 4).

This work is a step in the development of an effective clinical solution for the hemorrhage caused by *B. asper* snakebites and contributes for a better understanding of

neutralization of snake venom hemorrhagins by natural antidotes.

Acknowledgements

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